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Properties of the leak permeability induced by a cytotoxic protein from *Pseudomonas aeruginosa* (PACT) in rat erythrocytes and black lipid membranes

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A cytotoxic protein, isolated from *Pseudomonas aeruginosa* (PACT), was tested on red blood cells of rats and on black lipid membranes for changes of membrane permeability. In rat erythrocytes PACT induces lysis indicative of the formation of a leak permeable to monovalent ions. The dose response curve for the PACT-induced hemolysis demonstrates that the rate of lysis as well as the fraction of lytic cells increases with increasing toxin concentration. Furthermore, the leak pathway discriminates hydrophilic non-electrolytes according to their molecular weight. The findings indicate formation by PACT of a pore with an apparent radius of about 1.2 nm. In pure lipid membranes PACT forms hydrophilic pathways with moderate selectivity for small cations over small anions. The presence of cholesterol is a prerequisite for the occurrence of these PACT-induced permeability changes.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogenic microorganism of major importance in hospital-acquired infections, in particular in patients with reduced resistance [1–3]. For that reason it was tried to correlate clinical symptoms in patients with the appearance of various toxins from *P. aeruginosa* [4,5]. Scharmann [6] isolated a new cytotoxic protein, formerly called leucocidin and hereafter referred to as *P. aeruginosa* cytotoxin (PACT), that differed from several other toxins present in a variety of strains of *P. aeruginosa,* as reviewed in Refs. 7 and 8. This toxin with a molecular weight of 25 000 [9] preferentially causes damage to the distal tubule of the rat kidney [10]. In isolated perfused thick ascending limbs of Henle's loop of rabbit kidney it abolishes active NaCl transport within 1 min. This was interpreted by a primary increase of the permeability of the luminal membrane [11]. Similarly, a rapid loss of cell K^+ was demonstrated for isolated hepatocytes, AS-30D hepatoma cells [12], and for Ehrlich ascites tumor cells [13]. Simultaneously, cell Na^+ increased [13].

These rapid effects on cell cation leak fluxes prompted us to investigate the effect of PACT on a simple cell, the erythrocyte, and on artificial

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Abbreviations: PACT, *Pseudomonas aeruginosa* cytotoxin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; PBS, phosphate-buffered saline.

lipid membranes. Data on formation and some properties of the PACT-induced leak are presented in this study.

Materials

Chemicals were purchased from E. Merck, Darmstadt, F.R.G., unless mentioned otherwise.

Crude toxin was extracted from *Pseudomonas aeruginosa*, strain 158, as described by Scharmann [6], and purified according to Lutz [9]. The biological activities and biochemical properties of the batches used were identical to those described previously [10]. In SDS-polyacrylamide gel electrophoresis the protein appeared as a single band. Isoelectric focusing showed 4–6 components of comparable toxicity [9]. Rat blood was obtained from male Wistar rats (NMRI, Ges. für Versuchstierzucht, Hannover, weight 250–350 g) by portal vein puncture under ether- anesthesia after i.v. injection of about 8000 U.S.P. units Heparin- Na (Thrombophob®, Nordmark, Hamburg) per kg body weight. After centrifugation of the blood and removal of the buffy coat, erythrocytes were washed three times with isotonic saline.

Methods

(a) Hemolysis of cells in salt media containing toxin. Erythrocytes were suspended in a solution containing (mmol · l^{-1}): NaCl (137), KCl (2.68), $MgCl_2$ (0.5), KH_2PO_4 (1.43) and Na_2HPO_4 (3.93) (= PBS (pH 7.4), hematocrit 5%). Aliquots of the suspension were incubated with various concentrations of PACT at 37°C. After different times erythrocytes were sedimented by centrifugation. Following alkalinization of the supernatant with concentrated ammonia (10 µl/ml; 20 min) hemolysis was quantified by measuring the absorption of oxyhemoglobin at 546 nm.

(b)Hemolysis after pretreatment of cells with toxin followed by removal of non-membrane bound toxin. Erythrocytes were suspended in 10 vols of a medium containing (mmol · l^{-1}): KCl (90), NaCl (45), Na_2HPO_4/NaH_2PO_4 (1.25) and 25 mmol · l^{-1} (86 mosmol · l^{-1}) Dextran 4 (M_r 4000–6000, Serva, Heidelberg) to prevent hemolysis (medium A). Subsequently, PACT was added at various concentrations and the suspension incubated for 5 min at 37°C. After centrifugation, 1 vol. of erythrocytes was resuspended in 1 vol. of medium A. 40 µl of this suspension were mixed with 180 µl of a medium containing (mmol · l^{-1}): NaCl (45), KCl (90), Na_2HPO_4/NaH_2PO_4 (12.5) and 12 mmol · l^{-1} raffinose to retard hemolysis and facilitate its quantification. Hemolysis was registered continuously in a cuvette with 1 mm light path by the decrease of turbidity of the cell suspension at 700 nm.

Black lipid membranes. Solvent-containing black lipid membranes were obtained [14] from a 1–2% (w/v) solution of different lipids in *n*-decane (Fluka, Buchs, Switzerland). Solvent-free membranes from monolayers were formed as published earlier [15]. The membrane area was either 2 mm^2 (macroscopic measurements) for solvent-containing membranes or around 0.1 mm^2 (high current resolution) for solvent-free membranes. The aqueous solutions bathing the membrane were buffered with 10 mmol · l^{-1} phosphate at a pH of 7. The results were found to be pH-independent in the range of pH 5.5–7.5. In some experiments the toxin was already present prior to the formation of solvent-free and solvent-containing membranes, in others the toxin was added to preformed membranes under stirring.

The following lipids were used for membrane formation: egg phosphatidylcholine, egg phosphatidylethanolamine (purified by standard methods [16]), and cholesterol (Eastman, reagent grade). Oxidized cholesterol was prepared as described earlier [14].

For the electrical measurements the membrane cell was connected to a voltage source and a Keithley 610 C electrometer through Ag/AgCl electrodes. For the experiments with high current resolution a Keithley 427 preamplifier was used in connection with a Tektronix 5111/5A22 storage oscilloscope. The amplified signal was recorded with a strip-chart recorder. Zero-current membrane potentials were measured as described earlier [17]. Briefly, the membranes were formed in a 10^{-2} mol · l^{-1} salt solution in the presence of toxin. After the membrane had turned completely black the membrane conductance was observed to increase to a value which was considerably higher than the conductance of a pure membrane. The salt concentration on one side of the membrane

was then raised by the addition of small amounts of concentrated salt solutions. After about 10 min the zero-current membrane potential reached a steady-state value and was measured with a Keithley 610 C electrometer, using calomel electrodes with salt bridges.

Results

(a) Studies on rat erythrocytes

Incubation of rat erythrocytes in the presence of the cytotoxin from *Pseudomonas aeruginosa* (PACT) produces time- and concentration-dependent hemolysis (Fig. 1). The concentration of toxin not only determines the rate of lysis, but also the fraction of cells that undergo hemolysis. At a low amount of toxin per cell only a small fraction of cells becomes hemolytic, whereas at higher amounts of toxin this fraction gradually increases. Further experiments aimed to check the colloid-osmotic nature of hemolysis of erythrocytes by PACT. To this end erythrocytes were treated with toxin in salt solutions containing high-molecular-weight dextrans. Supplementation of the medium with 33 mosmol $\cdot l^{-1}$ Dextran 4 (M_r 4000–6000) fully suppressed the hemolytic action of PACT.

That dextran did not prevent leak formation by PACT could be demonstrated in the following way. Erythrocytes were pretreated with toxin in the presence of dextran, toxin removed by sedimentation of the cells by centrifugation, and the cells resuspended in a salt medium without dextran. As shown in Fig. 2, incubation of resuspended cells produced hemolysis at amounts of toxin per cell comparable to those in experiments in which toxin was present all the time. Furthermore, in pretreated cells, too, the fraction of hemolytic cells depends on the toxin concentration (Fig. 2). These results demonstrate the colloid-osmotic nature of toxin-induced hemolysis and the irreversibility of toxin binding to the cells.

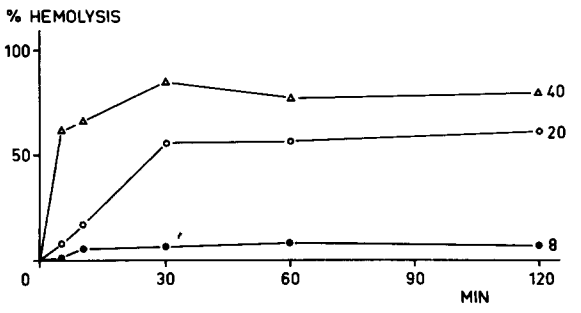

Fig. 1. Time-dependent hemolysis of erythrocytes in the presence of various amounts of PACT. Erythrocyte suspensions in phosphate-buffered saline (hematocrit 5%) were incubated at 37°C with 8, 20 or 40 μg of PACT/ml erythrocytes. Hemolysis, expressed as % of total hemoglobin in the suspension, was determined after increasing time intervals. See Methods for details.

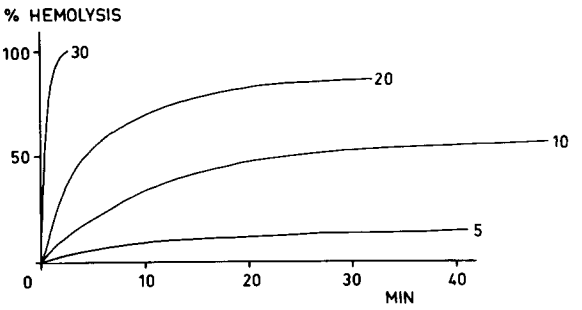

Fig. 2. Time-dependent hemolysis after pretreatment of erythrocytes with various amounts of toxin. Erythrocytes were treated with 5, 10, 20 or 30 μg of PACT per ml of cells, non-membrane bound toxin removed by centrifugation, cells resuspended in salt medium and hemolysis registered continuously. See Methods for details. Curves represent recorder tracings.

Interestingly, hemolysis of erythrocytes in presence of PACT (experimental conditions of Fig. 1) was found to depend on the anion composition of the incubation medium. The sequence of hemolysis rates in the presence of various ions was acetate $> Cl^- > Br^- > J^-$ (see also Ref. 18). In contrast, hemolysis of cells pretreated with toxin in NaCl medium (experimental conditions of Fig. 2), centrifuged, and resuspended in media containing different anions (F^-, Cl^-, J^-), did not demonstrate significant variations of hemolysis rates. It may be concluded therefore that the above mentioned dependence of hemolysis on the salt composition of the medium has to be due to differences in the binding of toxin and/or subsequent formation of the pore.

Further experiments with toxin-pretreated cells demonstrated a high temperature dependence of leak formation by PACT. Cells pretreated with PACT at 0°C in the presence of dextran, sedimented and resuspended in dextran-free medium

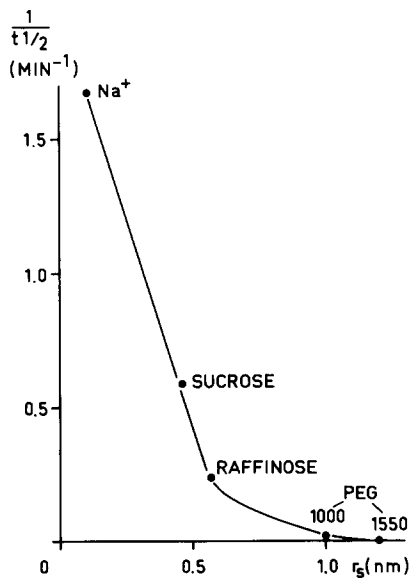

Fig. 3. Relationship between PACT-induced hemolysis of erythrocytes in the presence of various colloid-osmotic protectants and the molecular radii of these protectants. 1 vol. of erythrocytes was suspended in 2 vols. of medium A. Subsequently 150 μg PACT per ml of cells was added and the suspension incubated for 5 min at 37°C. After centrifugation the cells were resuspended in medium A at 50% hematocrit. 20 μl of this suspension were added to a medium containing 98 mmol · l^{-1} KCl, 49 mmol · l^{-1} NaCl, 2.5 mmol · l^{-1} Na_2HPO_4/NaH_2PO_4 and 33 ± 2 mosmol · l^{-1} of one of the test solutes. Time-dependent increase of hemolysis was continuously registered at 22°C as the decrease of turbidity at 700 nm. The reciprocal of half-times of hemolysis ($t_{1/2}$) was taken as a measure for the rate of hemolysis. Radii of non-electrolytes are viscometric radii, taken from Ref. 45.

did not hemolyze at PACT concentrations at which cells pretreated at 37°C hemolyzed within several minutes.

Variation of the time length of toxin pretreatment between 5 and 60 min did not significantly affect the rate of hemolysis. On the other hand, the extent of leak formation by PACT proved to be determined by the 'concentration' of toxin during the treatment and not by its 'amount per cell'. In a typical experiment, the amount of toxin required to hemolyse 50% of the cells at a hematocrit of 33% was 6.5 mg/ml of medium, equivalent to about 13 μg/ml of cells. At a hematocrit of 0.5% the same concentration was required (5.1 mg/l of medium; $n = 3$) which was now equivalent to an amount of toxin per ml of cells about 75-times higher than at the hematocrit of 33%. This lack of dependence of the effect on a certain amount of toxin per volume of cells suggests a simple (low-affinity) distribution of toxin between medium and membrane, and excludes high-affinity binding in the primary interaction of PACT with the cells.

In order to further characterize the pathway of leak permeability protective effects of different non-electrolytes of varying molecular weight, against colloid osmotic lysis of cells by PACT, were checked. This approach is based on the concept that colloid-osmotic lysis [19] can be suppressed by compounds, which are unable to penetrate the induced membrane leaks and are therefore capable of balancing the osmotic drag of intracellular impermeant solutes such as hemoglobin and organic phosphates. As shown in Fig. 3, protection against toxin-induced hemolysis was a function of the molecular weight of the non-electrolyte used as a protectant. In case of the low molecular weight substance sucrose, which does not penetrate into untreated erythrocytes, the rate of hemolysis, although retarded relative to lysis in isotonic NaCl solution, is still very high ($t_{1/2} = 2$ min). In the presence of poly(ethylene glycol) (PEG) 1550 ($r_s = 1.2$ nm) the rate of hemolysis is very low ($t_{1/2} > 100$ min). From these data (Fig. 3) it can be concluded that the pore radius is $\geqslant 1.2$ nm.

In order to assess possible effects of the toxin on cell morphology cells were studied by electron microscopy (Fig. 4). At increasing toxin concentrations the cells exhibit moderate swelling. Moreover, invaginations appeared, comparable to those observed in cells treated with cationic amphiphiles [20]. PACT may thus be classified as a stomatocytogenic agent. At high concentrations grossly distorted shapes (of ghosts?) were observed.

PACT bound to erythrocytes was just detectable by light microscopy using immunohistochemical techniques [10]. The binding was, however, too weak to be detectable by transmission electron microscopy in membrane sections. PACT 'bound' to erythrocyte membranes could also not be detected immunoelectrophoretically up to concentrations of 400 μg per ml erythrocytes.

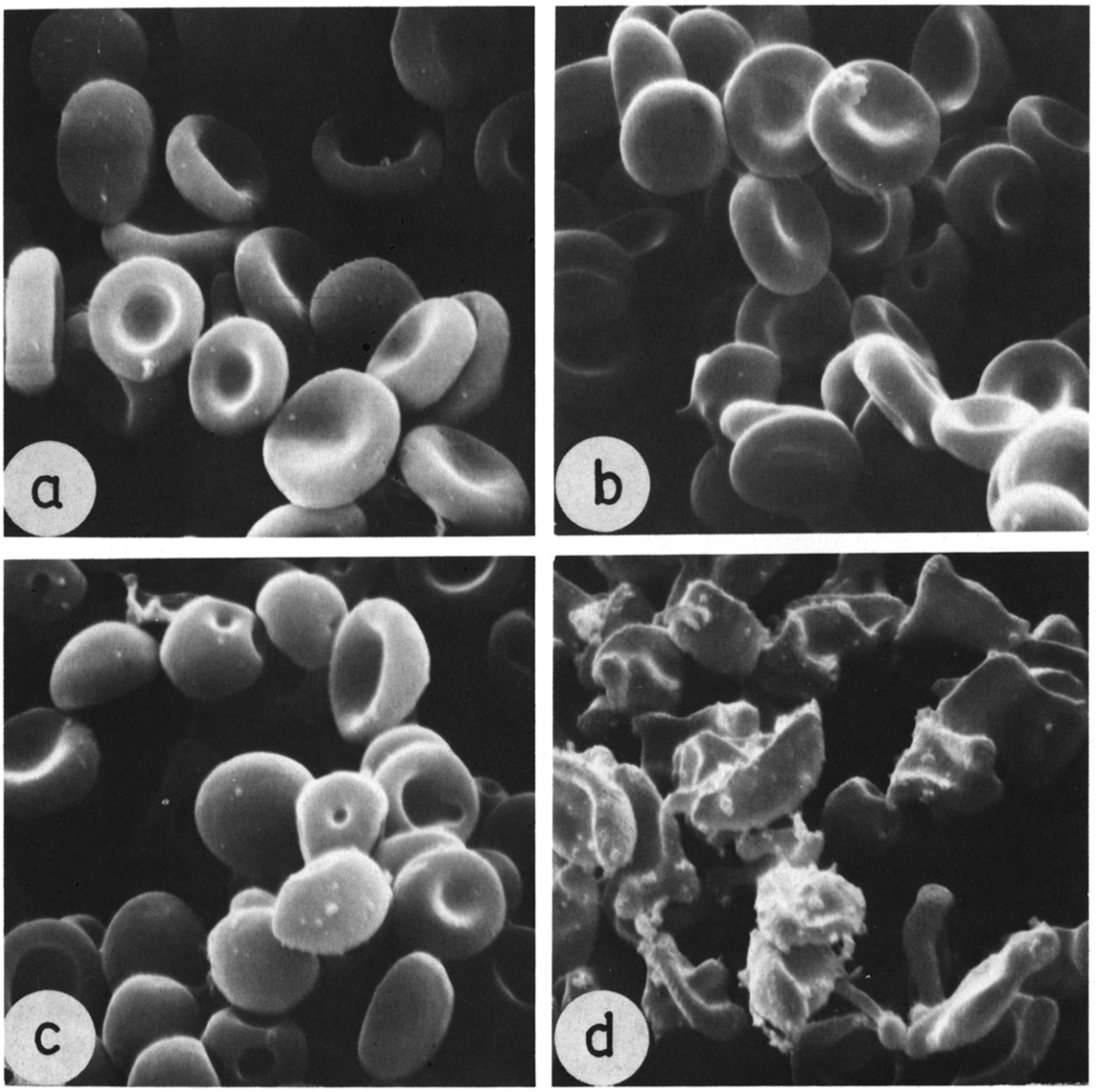

Fig. 4. Scanning electron micrographs of rat erythrocytes (×3700) treated with PACT. Erythrocytes were incubated for 30 min at 37°C with toxin at 60–100 (b), 100–300 (c), 400 (d) μg/ml of cells and in the absence of toxin (a) as described in Methods. Scanning electron microscopy was performed according to Burckhardt [46].

(b) Black lipid membranes

The interaction of PACT with artificial lipid bilayer membranes was studied by two approaches. In the first one PACT was added to preformed solvent-free or solvent-containing membranes prepared from different lipids or lipid/cholesterol mixtures. In this case only a minor increase of the conductance of the lipid bilayer was observed. An experiment of this type is shown in Fig. 5 (curve A) for a solvent-containing membrane from egg phosphatidylcholine/cholesterol (molar ratio 1 : 1). In the second approach membranes were prepared in the presence of the toxin. In contrast to preformed membranes an up to 1000-fold increase of conductivity occurred when solvent-free or solvent-containing membranes were formed in a PACT-containing solution (Fig. 5, curve B). The low amount of toxin needed to

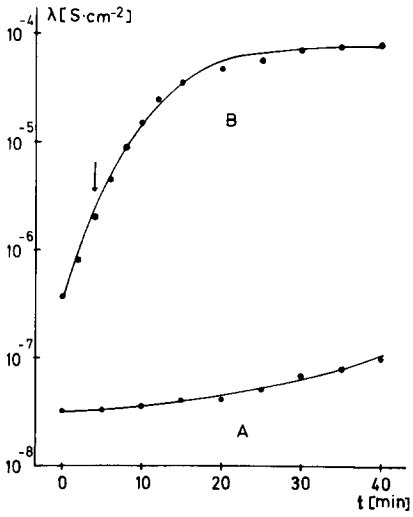

Fig. 5. Time-dependent conductivity changes of black lipid membranes in the presence of PACT. (A) PACT was added 5 min after formation of the black lipid membrane from egg phosphatidylcholine/cholesterol (molar ratio 1 : 1) in a final concentration of 0.4 μg/ml. (B) The membrane was formed in the presence of toxin (concentration as above). The arrow indicates the blackening of the membrane. The aqueous phase contained besides the toxin, 1 mol·l^{-1} KCl, 10 mmol·l^{-1} phosphate (pH 7.0), $T = 25$°C. The applied voltage was 10 mV in both cases.

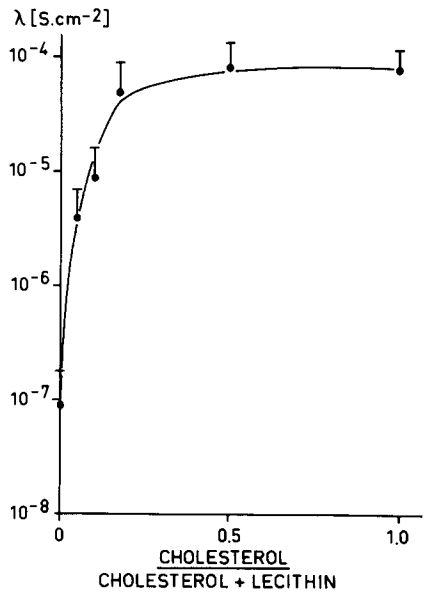

Fig. 6. Cholesterol dependence of increase of bilayer conductivity by PACT. Membranes were formed from various mixtures of egg phosphatidylcholine and cholesterol in an aqueous solution containing 1mol·l^{-1} KCl, 10 mmol·l^{-1} phosphate (pH 7.0) and 0.4 μg/ml PACT, $T = 25$°C. Conductance was measured 30 min after formation of the membranes at 10 mV. The data represent mean values (± S.D.) of five membranes.

obtain a conductance increase is comparable to that of other channel forming proteins, e.g. porins [38]. Moreover, conductance increase by porin in experiments with preformed membranes and membranes prepared in presence of porin [38] are of the same magnitude. This result may indicate lack of penetration of PACT into preformed membranes. Further experiments with solvent-free and solvent-containing membranes showed that cholesterol has to be present in lipid membranes for producing an increase of conductance (Fig. 6). A maximal effect was reached at a molar fraction of cholesterol of 0.15. This cholesterol dependence of the conductance increase by PACT in lipid membranes may be taken as an argument against leaks due to structural packing defects at the lipid-toxin interface, since such defects at interfaces between lipid and intercalated proteins can be sealed by cholesterol [47]. Although we observed a strong interaction between cholesterol-containing membranes and PACT, it was difficult to determine the dose-dependence of the PACT-induced increase of conductivity, because of the large scattering of the data at low toxin concentrations (40 ng/ml of medium). Nevertheless we obtained an approximately 10-fold lower conductance at a 10-fold lower toxin concentration (0.04 μg/ml as compared to 0.4 μg/ml), whereas 4 μg/ml PACT caused about the same conductance as 0.4 μg/ml PACT.

In another set of experiments the effect of the polar head group of the phospholipid on the interaction between PACT and membranes was investigated. Solvent-containing and solvent-free membranes were formed from mixtures of egg phosphatidylcholine and cholesterol, or egg phosphatidylethanolamine and cholesterol (molar ratio 1 : 1) in presence of PACT. Membrane conductance was measured as a function of time after bilayer formation. The conductance of bilayers containing phosphatidylethanolamine instead of egg phosphatidylcholine had comparable values, namely $(4 \pm 5) \cdot 10^{-5}$ and $(6 \pm 5) \cdot 10^{-5}$ S · cm^{-2}, respectively, ($n = 5$) 30 min after formation in the

presence of PACT. In membranes prepared from oxidized cholesterol in the presence of PACT an approximately 10-fold higher increase of conductance was observed within 30 min after formation as compared to membranes containing egg phosphatidylcholine/cholesterol (1 : 1). Furthermore we could not detect any major difference between PACT-induced effects on solvent-free and solvent-containing membranes. Experiments with high current resolution allow in principle to decide whether the single conductance unit of a transport system is a pore [21]. Therefore we performed a number of experiments of this type. It was not possible, however, to measure a defined single-channel conductance on cholesterol-containing membranes in the presence of PACT. A variety of conductance fluctuations was observed for 1 mol · l^{-1} KCl ranging from small conductance steps of 100 pS to steps with a conductance larger than several nS. In addition, a time-dependent increase of the current noise prevented detection of conductance fluctuations.

Zero-current potential measurements were used to check the ion selectivity of the PACT-induced pathway in cholesterol-containing membranes. The results are given in Table I. The potential is positive at the more diluted side with the small Cl^- anion and the small K^+ cation. This indicates a higher permeability for potassium than for chloride. If the small anion is replaced by a large ellipsoidal organic anion like $Hepes^-$ (axes 1.4, 0.6 and 0.5 nm) the zero-current potential is even more positive at the more diluted side.

In the case of a larger cation, $Tris^+$ (diameter 0.75 nm) and a small anion (Cl^-), the small anion is obviously better permeable since the potential is negative at the more diluted side. The zero-current potential as a function of the salt gradient could be very well explained by the Goldman-Hodgkin-Katz equation [17]. Calculations of the ratios of permeabilities for cations (c) over anions (a), P_c/P_a demonstrate a low selectivity of the hydrophilic pathway formed by the toxin for cations over anions in the case of small ions.

The permeability of large ions through the pathway seems to be low. This may be explained by the lower mobility of the large ions in the aqueous phase rather than by the assumption that large ions could not penetrate the pathway because of their large size. Otherwise the zero current potential should have a Nernstian slope, i.e. the zero-current potential for a 10-fold gradient should be either +59 mV or −59 mV at the more diluted side which would mean either $P_a/P_c = 0$ or $P_c/P_a = 0$, respectively.

TABLE I

ZERO-CURRENT POTENTIAL V_m IN THE PRESENCE OF A 10-FOLD SALT CONCENTRATION GRADIENT

Solvent-containing and solvent-free membranes were formed from a mixture of egg phosphatidylcholine/cholesterol (molar ratio 1:1) in an unbuffered aqueous solution (pH 7) in presence of 0.4 μg/ml PACT, $T = 25°C$. V_m is the electrical potential of the diluted side (30 mmol · l^{-1}) minus the potential of the concentrated side (300 mmol · l^{-1}). Ratios of permeabilities for cations (c) over anions (a), P_c/P_a were calculated from the Goldman-Hodgkin-Katz-equation as described previously [17]. The mean values (±S.D.) of three membranes are shown.

Salt	V_m (mV)	P_c/P_a
KCl	19±4	2.5±0.6
$Tris^+$ Cl^-	−31±3	0.2±0.1
K^+ $Hepes^-$	30±4	4.2±0.7

Discussion

Pseudomonas aeruginosa cytotoxin (PACT) exerts a nonspecific lytic effect on a great variety of cells [21]. The sensitivity of different cell types is very divergent, polymorphonuclear leucocytes being among the most sensitive. Moreover, even among erythrocytes from various animal species sensitivity to lysis is highly variable. Erythrocytes from rats are much more sensitive than human erythrocytes. The mechanism of the lytic process has not been fully elucidated thus far. Cell lysis is claimed to be preceded by loss of intracellular K^+ and swelling of the cells [22]. Recently, PACT has been shown to produce an up to 10-fold increase of the conductance of preformed lipid bilayer membranes of oxidized cholesterol [13].

In the work presented here we have demonstrated that artificial lipid bilayers and the erythrocyte membrane respond to the addition of toxin in a different way. Addition of toxin to lipid membranes results in a minor, time-dependent increase of membrane permeability to small ions, which was also observed for membranes without

toxin. In erythrocytes, however, addition of toxin leads to pronounced leak formation. On the other hand, PACT increases lipid bilayer conductivity 1000-fold when the bilayer is formed in the presence of the toxin. A comparison of the effects of added PACT on the erythrocyte membrane and on the lipid bilayer suggests that the toxin needs a binding site for formation of the leak in biomembranes. A possible role of membrane proteins in binding of the toxin is supported by the large variation of the sensitivities of different cell types. A marked resistance of *Acholeplasma laidlawii* to PACT [23] is also in line with such a concept. Under the conditions of cultivation of *Acholeplasma* the molar ratio of cholesterol to phospholipid in the membrane was 0.5. At this ratio PACT produces maximal effects in lipid membranes (cf. Fig. 6). A further indication that proteins are involved in the primary interaction of the toxin with the membrane comes from studies on the effect of proteolytic digestion of surface proteins on toxin-induced hemolysis. Pretreatment of erythrocytes with trypsin (1 mg/ml, 30 min, 37°C) followed by 5 washings of the cells and addition of trypsin inhibitor (0.5–1 mg/ml of medium, 10% hematocrit prevents toxin-induced hemolysis (Weiner and Haest, data not shown). This treatment is known to remove sialo- and other glycopeptide residues from the outer membrane surface [24]. The dependence of the extent of lysis on the toxin concentration rather than on the amount of toxin available per cell is also reconcible with a reversible equilibrium binding of the toxin (to proteins?) as the primary step in toxin action on the membrane.

The observation that at lower toxin concentrations only a certain fraction of the cells undergoes lysis indicates that the population of erythrocytes is inhomogeneous with respect to toxin sensitivity. Such differences in toxin-sensitivity between the cells of an erythrocyte population might for instance be due to age-related variations in the carbohydrate composition of membrane glycoproteins [25–28]. From studies on lipid bilayers it is clear that the toxin can be inserted into the hydrophobic core of a membrane after its primary binding to the cell surface. An interaction of the toxin with the lipid bilayer may also be derived from the observed enhancement of transbilayer reorientation rates of phospholipids in the erythrocyte membrane [29] after addition of PACT.

The leaks induced by the toxin in rat erythrocytes may be visualized as aqueous pores with an apparent radius $r_p \geq 1.2$ nm in view of their sieving properties (Fig. 3). A very rough calculation also provides equivalent numbers of pores per cell. A permeability coefficient for the salts taken up (NaCl, KCl) in the course of prelytic cell swelling can be calculated from the $t_{1/2}$ for lysis, by formulae originally given by Jacobs [30]. From this permeability coefficient a total area available for leak diffusion in toxin-treated cells can be estimated assuming diffusion coefficients in the pore equal to those in bulk solution and a pore length of 5 nm (the thickness of the hydrophobic membrane core). From the total area and the apparent area per pore (πr_p^2) an apparent number of pores is finally obtained. The data in Fig. 3 ($r_p \geq 1.2$ nm, $t_{1/2}$ for lysis in NaCl 0.6 min) indicate an equivalent number of pores of about 1 per cell. Since a considerable number of crucial assumptions is part of these calculations the results should only be taken to indicate that a very small number of PACT-induced defects will account for the rapid lysis of the cells.

In view of the enhancement of transbilayer phospholipid mobility by PACT these defects might result from packing disorders at the toxin/lipid interface after insertion of PACT into the hydrophobic membrane core. Such defects at a lipid/protein interface, acting as aqueous pores with an apparent radius between 0.6 and 3 nm have also been postulated for lipid vesicles containing intrinsic membrane proteins [31,32].

Alternatively, the leak pathway might be formed by aggregates of toxin monomers as claimed for a number of other toxins [33–35], serum complement [36,37], bacterial and mitochondrial porins [38,39].

For a number of membrane leaks induced by exogeneous proteins inserted into membranes, defined single-unit conductances have been observed which suggest that indeed defined aggregates are formed [34,35,40–43].

In case of PACT, conductance fluctuations of widely varying height were observed. It remains to be clarified whether such a finding indicates formation of fluctuating defects varying in size, space

and time at lipid/protein interfaces. The apparent pore number of 1 per cell may be in line with such a concept since it seems rather unlikely the toxin material equivalent to only one leak site will be bound per cell. The ion selectivity among small ions ($P_c/P_a = 2.5:1$; black lipid membranes) can probably not be used for a distinction between the alternative models of the defect, since such low selectivities have been described for well-defined pores [34,35,38,40,43] as well as for undefined leaks created e.g. by chemical modification of the erythrocyte membrane [44]. More detailed work on the PACT-induced leak will be required to establish its structural nature.

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